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Evidence that *nervy*, the *Drosophila* homolog of ETO/MTG8, promotes mechanosensory organ development by enhancing Notch signaling

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Abstract

In the imaginal tissue of developing fruit flies, *achaete* (*ac*) and *scute* (*sc*) expression defines a group of neurally-competent cells called the proneural cluster (PNC). From the PNC, a single cell, the sensory organ precursor (SOP), is selected as the adult mechanosensory organ precursor. The SOP expresses high levels of *ac* and *sc* and sends a strong Delta (Dl) signal, which activates the Notch (N) receptor in neighboring cells, preventing them from also adopting a neural fate. Previous work has determined how *ac* and *sc* expression in the PNC and SOP is regulated, but less is known about SOP-specific factors that promote SOP fate. Here, we describe the role of *nervy* (*nvy*), the *Drosophila* homolog of the mammalian proto-oncogene *ETO*, in mechanosensory organ formation. Nvy is specifically expressed in the SOP, where it interacts with the Ac and Sc DNA binding partner Daughterless (Da) and affects the expression of Ac and Sc targets. *nvy* loss- and gain-of-function experiments suggest that *nvy* reinforces, but is not absolutely required for, the SOP fate. We propose a model in which *nvy* acts downstream of *ac* and *sc* to promote the SOP fate by transiently strengthening the Dl signal emanating from the SOP.

Keywords: *nervy*; *ETO*; *MTG8*; Neurogenesis; *Drosophila*; Delta; Daughterless; Achaete; Scute; Proneural genes; Notch

Introduction

The body of the adult fruit fly is covered by bristles that function as chemo- and mechano-sensory organs, enabling the fly to sense and navigate its environment. These bristles, called chaetae, comprise part of the fly's peripheral nervous system. Each mechanosensory organ consists of 4 cells: the external bristle and socket cells and the internal neuron and sheath. These 4 cells are all derived from a single cell, the SOP.

The development of the adult *Drosophila* mechanosensory organs is initiated by the expression of bHLH transcription factors encoded by *ac* and *sc* (reviewed in Gomez-Skarmeta et al., 2003). In larval imaginal discs, *ac* and *sc* are first expressed in clusters of approximately 15–20 cells at positions corresponding to the location of bristles in the adult fly. *ac* and *sc* are part of the *achaete scute c*omplex (*AS-C*), which has two other members: *lethal of scute*, which is not expressed in imaginal discs, and *asense* (*ase*), which is expressed in the SOP after it differentiates (Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994; Jarman et al., 1993). Two types of enhancers mediate the expression of *ac* and *sc* during the process of SOP formation (Gomez-Skarmeta et al., 2003). One type of enhancer (called a PNC enhancer) integrates positional information within the imaginal disc and results in low levels of *ac* and *sc* expression throughout the PNC, including the presumptive SOP. Although *ac* and *sc* are at first uniformly expressed in the PNC, their expression becomes elevated in one or two cells of the cluster, the future SOPs (Cubas et al., 1991; Culi and Modolell, 1998; Huang et al., 1991). High *ac* and *sc* expression in the nascent SOP is mediated by a separate enhancer, referred to as a SOP enhancer. Studies of the *sc* SOP enhancer revealed that Sc directly and positively regulates its own expression within the SOP (Culi and Modolell, 1998).

The increase in *ac*/*sc* in the SOPs is thought to lead to an increase in the activity of the N ligand Dl, which activates the N pathway in neighboring PNC cells (Heitzler and Simpson,

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1991; Rooke and Xu, 1998; Simpson, 1997). N activity results in the expression of Enhancer of Split (E(spl)), which represses *ac* and *sc* expression mediated by the SOP enhancer in the epidermal-fated cells of the PNC (Bailey and Posakony, 1995; Culi and Modolell, 1998; Giagtzoglou et al., 2003; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). Thus, the SOPs signal through Dl to prevent other cells in the PNC from adopting a neural fate and the activity of the SOP enhancer is restricted so that only a few cells – the future SOPs – accumulate high levels of Ac and Sc.

Although *ac* and *sc* are required for the initial specification of the SOP, they are dispensable for later stages of mechanosensory organ development. Accordingly, *ac* and *sc* expression decreases and is no longer detectable when the SOP undergoes its first division (Cubas et al., 1991). Instead, other factors that are activated by *ac* and *sc* are thought to continue the process of SOP differentiation. One of these factors is *senseless* (*sens*), a direct target of *ac* and *sc* that is both necessary and sufficient for SOP specification (Jafar-Nejad et al., 2003; Nolo et al., 2000).

A critical step in the selection of the SOP occurs when Ac/Sc levels increase, leading to an increase in the strength of the inhibitory signal sent to neighboring cells via the N pathway. Despite the importance of this step, little is known about how Dl activity is enhanced in the presumptive SOP. Although it is likely that *ac*/*sc* target genes play a role in this process, direct evidence for this idea is lacking (Heitzler et al., 1996). Here, we describe the role of *nervy* (*nvy*), the *Drosophila* homolog of the human proto-oncogene *ETO*, in sensory organ development (Feinstein et al., 1995). *nvy* encodes a nuclear protein that is specifically expressed in SOPs where it interacts with the Ac and Sc binding partner Da. Based on the functional domains it shares with ETO, Nvy is likely to interact with transcriptional co-repressor complexes (reviewed in Davis et al., 2003). Consistent with this notion, we show that Nvy can repress the activity of enhancers that are normally activated by Ac/Sc/Da complexes, including an SOP enhancer. Furthermore, genetic analysis of mosaic nvy^{-} tissue suggests that although *nvy* is not essential for SOP specification, nvy^{+} activity biases a neurally competent PNC cell to become an SOP. We also show that *nvy* genetically interacts with components of the *N* pathway and that an activator form of Nvy can reduce Dl levels. Based on these results, we suggest a model in which Nvy normally represses the expression of a factor that downregulates Dl activity, thus promoting the SOP fate.

Materials and methods

Fly stocks and crosses

DC-lacZ, *SOP-lacZ* (also called *SRV-lacZ*), *E-lacZ*, *mE-lacZ*, *UAS-sc*, *UAS-da*, *UAS-ase*, and sca^{C253}*-Gal4* were generously provided by the Modolell lab; *UAS-sens* was shared by H. Bellen; N^{XK11} (a null allele of *N*) was the gift of G. Struhl; Dl^{CS} was shared by J. Posakony; $Dl^{A326.2F3}$ (also known as $Dl^{P\text{-}lacZ}$) was kindly supplied by M. Muskavitch; da^{10} *FRT 40A* is a null allele of *da* obtained from the Bloomington Stock Center. The *nicastrin* mutation was described in (Chung and Struhl, 2001) and was provided by G. Struhl. UAS-*nvy* was made by cloning *nvy* cDNA (Feinstein et al., 1995) into *pUASt*. To create *UAS-VP16-nvy* the *VP16* activation domain was fused in frame to the 5′ end of *nvy* using a *Bam*HI site in the *nvy* cDNA. *UAS-nvyΔZF* was created by PCR amplifying the DNA flanking the zinc fingers and fusing the PCR products together (this removes amino acids 575–625). The *UAS-nvy-RNAi* construct is a cDNA/genomic DNA hybrid created using the guidelines previously described (Kalidas and Smith, 2002). Nucleotides 1314–1889 of the *nvy* cDNA were fused to the complimentary *nvy* genomic region and cloned into *pUASt*. nvy^{PDFKG1} and neutral clones were generated in flies of the following genotypes: *yw hs-flp*; *G13* nvy^{PDFKG1}*/G13 Ubi-GFP* and *yw hs-flp*; *G13* y^{+}*/G13 Ubi-GFP*, respectively. *nvy* RNAi clones were generated in *yw hs-flp*; *UAS-nvy-RNAi/+*; *tub>y+ GFP>Gal4 UAS-nvy-RNAi* flies; clones ectopically expressing *nvy* were generated in *yw hs-flp*; *UAS-nvy*; *tub>y+ GFP>Gal4* flies and neutral clones were generated in *yw hs-flp*; *tub>y+ GFP>Gal4* flies. The clones ectopically expressing *nvy* were marked by the absence of GFP, and the distance from clone border to SOP was measured by counting the number of interceding GFP+ cells. A Fisher's exact test was used to analyze the number of SOPs located near clones of ectopic *nvy*, nvy^{PDFKG1} mutant clones and *nvy* RNAi clones. The number of SC bristles in the different genetic backgrounds described in Tables 3 and 4 were compared using a *t* test (the number of DC bristles and microchaetae density in these genetic interaction tests were not significantly affected). *pnr-Gal4* (pnr^{MD237}) is a hypomorphic allele of *pnr*. Because *pnr* directly activates *DC-lacZ*, we compared the expression of *DC-lacZ* in *pnr-Gal4 UAS-nvy* flies to *pnr-Gal4* flies.

Immunofluorescence and antibodies

Dissected tissue was prepared by standard procedures (for anti-Ac and anti-GFP stains, discs were fixed in 4% methanol-free formaldehyde (Polysciences, Inc)). The following primary antibodies were used at the indicated dilution: anti-Sens, 1/1000 (H. Bellen); anti-βgal, 1/2000 (Cappell); anti-GFP, 1/1000 (Molecular Probes); anti-Elav, 1/50; anti-Ac, 1/2; anti-Dl, 1/1000 and 22C10, 1/10 (Hybridoma Bank). A polyclonal antibody was raised against GST-Nvy in rabbits (Cocalico Biologicals, Inc., Reamstown, PA). Anti-Nvy recognizes a band of the predicted size on Western blots and does not recognize any antigen in nvy^{-} embryos or nvy^{-} clones, indicating that the antibody specifically recognizes Nvy. Anti-Nvy was preabsorbed and used at a 1/300 dilution to probe wild type tissue and 1/600 to probe tissue ectopically expressing Nvy.

GST pull-downs

GST-Nvy was created by cloning *nvy* cDNA into *pGEX*. GST and GST-Nvy were expressed in bacterial cells, which were lysed to produce bacterial cell extract. Embryonic lysate was prepared by homogenizing 600 μL of dechorinated wild type embryos in 900 μL 1× PBS, 1% Triton X100 and protease inhibitors. His-Da was made by cloning *da* cDNA (kind gift of S. Campuzano) into *pET14b*. Bacterially expressed His-Da was purified under denaturing conditions using Ni^{2+}-NTA beads (Qiagen). For each pull-down reaction 500 μL of GST or GST-Nvy bacterial cell extract was incubated with either 500 μL of embryonic lysate or 6 pmol of purified His-Da. The reactions were separated on an SDS-PAGE gel and probed with mouse anti-Da (1/500; kindly provided by C. Cronmiller). Using anti-Da to probe material from animals over-expressing Da shows an enrichment of a band of the predicted size of Da; anti-Da also recognizes in vitro translated Da protein (data not shown).

Results

Ectopic nvy blocks mechanosensory organ formation

nvy is widely expressed in the developing nervous system (Feinstein et al., 1995). To initially assess its role in nervous system development, we used the Gal4 UAS method to ectopically express Nvy during development. We used the *pannier-Gal4* (*pnr-Gal4*) driver, which is expressed in the dorsal region of the fly where there are the dorsocentral (DC)

and scutellar (SC) macrochaetae as well as many microchaetae. Surprisingly, expressing Nvy in this manner completely suppressed sensory organ formation. The dorsal region of *pnr-Gal4 UAS-nvy* flies is 'bald,' and lacks both the external and internal components of these sensory organs (Figs. 1B and C, and data not shown). The loss of mechanosensory organs is not due to cell death, as co-expression of the cell death inhibitor p35 had no effect on the ectopic *nvy* phenotype (data not shown). This effect of ectopic *nvy* on sensory organ development is not specific to mechanosensory organs on the notum, as ectopically expressing *nvy* using several other Gal4 drivers (e.g., *tubulin-Gal4*, *scabrous-Gal4*, *decapentaplegic-Gal4*) resulted in flies that have 'bald' legs, wing margins, abdomens, and eyes (data not shown). Besides a block of sensory organ development, no other morphological affects were observed following ectopic Nvy expression.

nvy is widely expressed in developing embryonic and adult nervous systems

To begin to understand the ectopic Nvy phenotype, we better characterized its expression pattern during *Drosophila* development using an anti-Nvy antibody. At all stages of development, Nvy was observed in nuclei (see also Ice et al., 2005). In the embryo, Nvy was initially detected in delaminating neuroblasts, the precursors of the embryonic nervous system, and their progeny, the ganglion mother cells (gmc) (Figs. 1D, E). In older embryos, Nvy was widely expressed in the mature embryonic CNS and PNS (Figs. 1F, G).

During adult mechanosensory organ development, *nvy* was expressed in singled-out SOPs, but was not detected in the surrounding cells of the PNC (Fig. 1H). The onset of *nvy* expression in the SOP is coincident with that of *sens*, which is a direct target of *ac* and *sc* (Jafar-Nejad et al., 2003). Nvy was initially detected when higher levels of Ac and Sc accumulate in the newly-formed SOPs, suggesting that *nvy* is also a target of *ac* and *sc* (Figs. 1H, J, K). Consistently, ectopic *sc* results in extra SOPs that also express *nvy* (data not shown). As *nvy* expression in the SOPs increases, there is a corresponding decrease in *ac* expression (Figs. 1J, K). The SOP and its progeny can be identified by the expression of *neuralized* (*neur*)*-Gal4 UAS-GFP*. Using this marker, we found that *nvy* expression is maintained as the SOP and its progeny divide, and that *nvy* is expressed in the neuron, sheath, socket, bristle and glia (the glia dies shortly after it forms (Fichelson and Gho, 2003); Fig. 1I). *nvy* is also expressed in the developing chordotonal organs, eye, chemosensory organs in the antenna, and a subset of neurons in the ventral nerve cord and brain (Figs. 1L, M, and data not shown).

Ectopic Nvy suppresses the formation of the SOP but not the PNC

The *pnr-Gal4 UAS-nvy* experiments described above are paradoxical because although ectopic Nvy suppresses sensory organ development, *nvy* is normally expressed in the SOP and its progeny. To address this paradox, we examined *pnr-Gal4 UAS-nvy* imaginal wing discs to determine if the DC and SC PNCs and SOPs form normally. *pnr-Gal4* is active in the dorso-medial domain of the wing disc prior to and during PNC and SOP formation. We utilized an anti-Ac antibody and a *DC-lacZ* reporter gene to mark the DC PNC (Garcia-Garcia et al., 1999), and the *sc SOP-lacZ* reporter gene that is expressed in the singled out SOP but not other PNC cells (Culi and Modolell, 1998).

In *pnr-Gal4 UAS-nvy* wing discs the expression of *ac* in the PNC and *DC-lacZ* appeared wild type (Figs. 2F, G). These results suggest that *pnr>nvy* expression does not interfere with PNC formation. In contrast, ectopic Nvy completely repressed *SOP-lacZ* within the *pnr* domain and inhibited the upregulation of *ac* in the nascent SOP (Figs. 2F, H). Thus, the lack of sensory organs in *pnr-Gal4 UAS-nvy* flies is not due to a loss of neural competence within the PNC. Instead, these results demonstrate that ectopic *nvy* blocks the specification of the SOP. In support of this idea, the expression of another SOP marker, Sens, is absent in *pnr-Gal4 UAS-nvy* nota (see Fig. 5F below).

The loss of SOPs in *pnr-Gal4 UAS-nvy* flies could be the result of either higher levels of *nvy* (in the SOP or PNC) or precocious *nvy* expression. To distinguish between these possibilities, we used *neur-Gal4*, which is active specifically in SOPs, to increase *nvy* expression at the time and place where *nvy* is normally expressed. *neur-Gal4 UAS-nvy* flies appear wild type, suggesting that higher levels of Nvy in the SOP do not interfere with SOP differentiation (Fig. 2I). We also generated flip-out clones expressing Nvy. These clones show that forcing Nvy expression in non-SOP PNC cells does not interfere with SOP formation (although it does affect the SOP position, see below) (Fig. 2J). Thus, the *pnr-Gal4 UAS-nvy* phenotype is more likely a result of widespread and precocious *nvy* expression, rather than an increase in *nvy* expression in the SOP.

nvy loss-of-function suggests that nvy influences N signaling

The loss of SOPs in *pnr-G4 UAS-nvy* flies is reminiscent of gain-of-function phenotypes in the N pathway, suggesting that *nvy* may be affecting N signaling. To test this, we utilized a small deficiency that deletes *nvy* (nvy^{PDFKG1}; Terman and Kolodkin, 2004) and a *nvy* RNAi construct to analyze loss-of-function phenotypes in mosaic flies. In most cases, homozygous nvy^{PDFKG1} clones generated sensory organs with the wild type complement of cell types, suggesting that *nvy* is not required for specifying the SOP or the fate of its progeny (data not shown). However, as described below, the *nvy* genotype affected a cell's propensity to become an SOP.

Previous studies have shown that differences in the relative level of N activity between neighboring cells biases which cell adopts the SOP fate (Heitzler and Simpson, 1991). For example, a cell with more copies of Dl^+ has a greater tendency to adopt a SOP fate when it neighbors a cell with less Dl^+. If *nvy* affects N signaling, we predict that when the boundary of a *nvy* mutant clone falls within a PNC that the absence of *nvy* should bias the SOP to preferentially form on one side of the

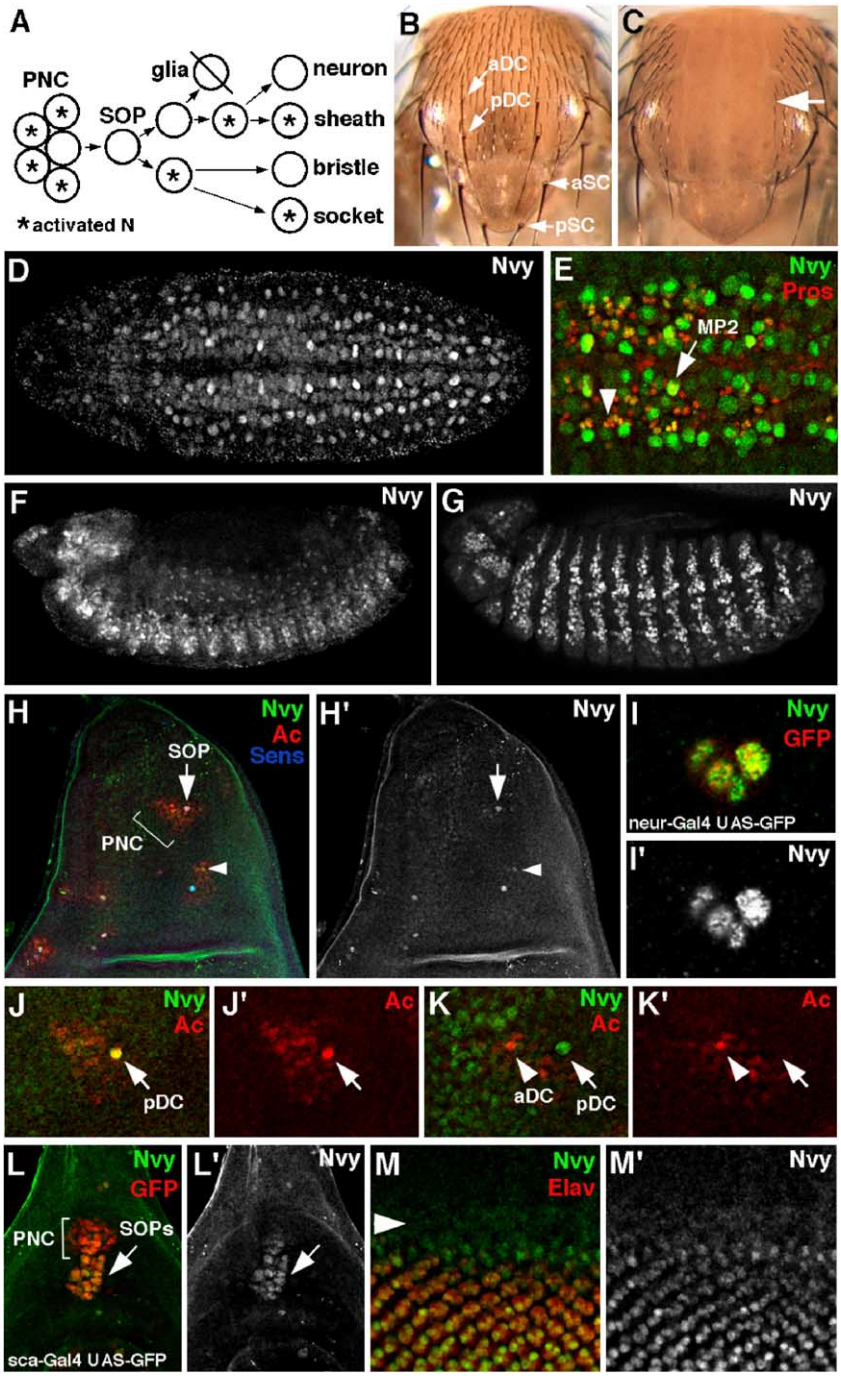

Fig. 1. Although *nvy* is widely expressed in the developing fly nervous system, ectopic *nvy* inhibits mechanosensory organ formation. An anti-Nvy antibody (green or white) shows the pattern of *nvy* expression in the developing fly nervous system. Nvy is only detected in nuclei with this antibody. (A) Cartoon of mechanosensory organ development. The SOP is selected from the PNC and divides to give rise to a glia (which dies, indicated by a slash), neuron, sheath, bristle, and socket. Dl-N signaling is active at each step, first acting to select a single SOP from the PNC and promoting binary cell fate decisions in the SOP lineage (* denotes a cell with activated N). (B) The back of a wild type fly. The anterior (a) and posterior (p) DC and SC macrochaetae are indicated. (C) The *pnr* domain of *pnr-Gal4 UAS-nvy* flies is bald (arrow). (D) During embryogenesis, Nvy is first detected in delaminating neuroblasts. (E) Nvy expression levels vary among the neuroblasts. For example, Nvy levels are high in the MP2 neuroblast (arrow). Nvy is also expressed in the gmc (arrowhead), which express Prospero (red). (F, G) Nvy is widely expressed in the embryonic CNS (F) and PNS (G). (H) Ac (red) is expressed in both PNCs (bracket) and SOPs (arrow) in the larval wing disc. Nvy is expressed specifically in SOPs, which are marked by Sens (blue). The arrowhead points to a presumptive SOP with high levels of Ac, low levels of Nvy but does not yet express Sens. (I) Nvy is observed in all four cells of the mature mechanosensory organ in a pupal notum, marked by *neur-Gal4 UAS-GFP* (red). (J) Within the DC PNC the pDC SOP (arrow) expresses both Ac (red) and Nvy. (K) Slightly later, the aDC SOP (arrowhead) has differentiated and strongly expresses Ac but only weakly expresses Nvy. At this time Ac is no longer expressed in the older pDC SOP (arrow). Nvy is also expressed in the peripodium, and Nvy-expressing peripodial cells are visible in the left of the photo. (L) In developing chordotonal organs (which express *sca-Gal4 UAS-GFP*; red) Nvy is present in SOPs (arrow) but not the PNC (bracket). (M) Posterior to the morphogenetic furrow (arrowhead) in the larval eye disc, Nvy is observed in the differentiating photoreceptors, which express Elav (red).

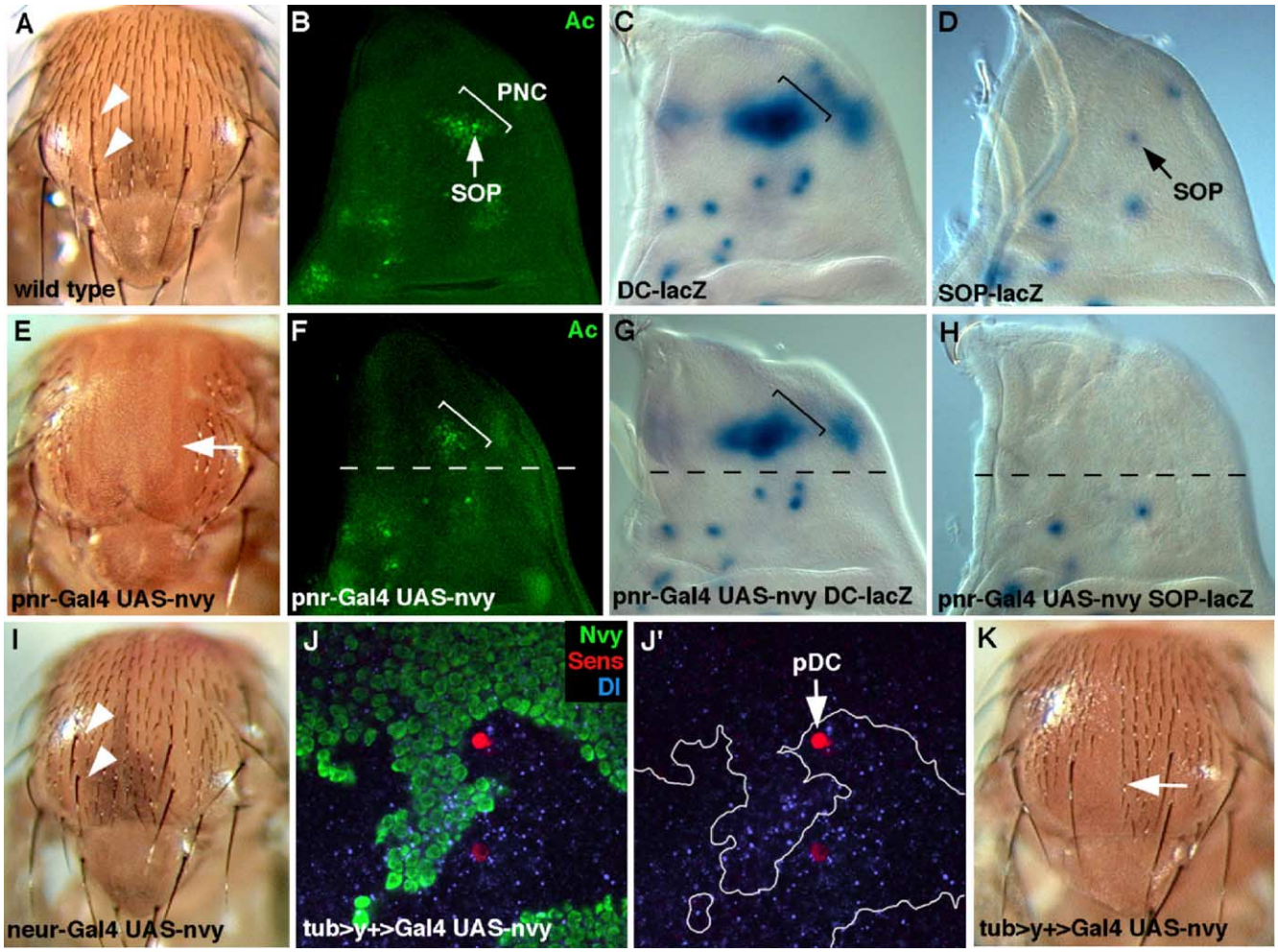

Fig. 2. Ectopic *nvy* inhibits mechanosensory organ formation by blocking SOP formation. Arrowheads mark the DC bristles and brackets denote the DC PNC. The dashed line (F–H) marks the approximate limit of the *pnr* domain in the larval wing disc. (A, E) The back of wild type (A) and *pnr-Gal4 UAS-nvy* (E) adult flies. The DC bristles and other macrochaetae and microchaetae in the *pnr* domain are missing in the *pnr-Gal4 UAS-nvy* flies (arrow). (B, F) *ac* expression (green) in wild type (B) and *pnr-Gal4 UAS-nvy* (F) larval wing discs. (C, G) X-gal stain showing wild type *DC-lacZ* expression (C) and *DC-lacZ* expression in *pnr-Gal4 UAS-nvy* (G) larval wing discs. (D, H) X-gal stain showing *SOP-lacZ* expression in wild type (D) and *pnr-Gal4 UAS-nvy* (H) larval wing discs. There is no *SOP-lacZ* expression in the *pnr* domain of *pnr-Gal4 UAS-nvy* wing discs. (I) *neur-Gal4 UAS-nvy* adult flies appear wild type. (J) A clone of ectopic Nvy (green) in the larval wing disc. This clone covers the anterior part of the DC PNC, which usually gives rise to the aDC SOP (the SOPs are marked by Sens in red). Outside the clone, the pDC SOP forms normally, but within the clone, the aDC SOP is missing. The clone border is outlined in the separated channel. The tissue is also stained for Dl (purple). (K) A clone ectopically expressing Nvy results in bald patches (arrow) in the adult.

clone border. We tested this idea in two ways. First, we generated nvy^{PDFKG1} clones and scored the position of the SOPs that formed near the clone borders in mid-third instar larval discs, shortly after SOPs differentiate. Second, because nvy^{PDFKG1} removes more than just *nvy* (Terman and Kolodkin, 2004), we also analyzed the position of SOPs relative to the border of clones that express an RNAi construct that targets Nvy (*nvy-RNAi*). In both cases, nvy^{+} PNC cells are more likely to adopt the SOP fate than nvy^{-} PNC cells (Table 1). The bias we observed is similar to that reported for PNC cells that have a greater dose of Dl^{+} (Heitzler and Simpson, 1991). These results suggest that *nvy*-expressing cells are more likely to adopt the SOP fate than are *nvy*-non-expressing cells.

One way in which nvy^{+} activity could bias cells in favor of the SOP fate would be to increase the strength of the Dl signal sent to neighboring cells. If this were the case, we would predict that cells in which we force Nvy expression should inhibit their immediate neighbors from adopting the SOP fate. To test this idea, we asked where SOPs form relative to the boundary of clones that ectopically express Nvy. Consistent with our previous experiments, SOPs are never observed within these clones and, as expected, they resulted in bald patches of cuticle in the adult (Fig. 2K). When we compared the frequency of SOPs that touch the ectopic Nvy clone versus SOPs that form one to two cells away from the clone, we found that SOPs were more likely to form one to two cells away from the border of Nvy^{+} clones as compared to control clones (Table 2). These results suggest that Nvy expression can non-autonomously inhibit SOP formation, consistent with the idea that nvy^{+} activity increases the strength of the Dl signal.

Consistent with the above analysis, we also found that *nvy* and *N* genetically interact. In *N* heterozygous mutant flies the average number of SC macrochaetae is slightly increased over WT. Decreasing *nvy* levels in heterozygous *N* mutant flies

Table 1
Position of SOPs relative to the boundaries of neutral and *nvy*$^-$ clones in wing discs

	# SOPs outside the clone	# SOPs inside the clone	*n*	% SOPs outside the clone
neutral FRT clone	40	43	83	48.2
nvy^{PDFKG1} clone	56	26	82	68.3*
tub > GFP > Gal4 clone	52	33	85	61.2
tub > GFP > Gal4 UAS-nvy-RNAi clone	71	20	91	78.0*

SOPs located at clone borders were scored if the SOP was located inside or outside the clone (*n* = number of SOPs scored). Significantly more of the SOPs present at *nvy*$^-$ clone borders were located outside the clone (*0.05 > *p*). Only SOPs whose neighbors could be unambiguously identified were counted. The following SOPs were scored: pSC, aSC, pDC, aDC, tr1, aPA, pSA, pNP, aNP and a portion of the tegula SOPs not in the hinge region (SOPs located along the hinge and costa, where the epithelia is folded, were not included. SOPs along the wing margin were also excluded).

significantly increased the number of SC bristles even further (Table 3). These results suggest that *nvy* stimulates the activity of a component of the N signaling pathway. Although *nvy*^{PDFKG1} did not interact with a null allele of *Dl* (*Dl*^{B2}), removing one copy of *nvy* did significantly increase the number of SC bristles in *Dl*^{CS} mutant flies (Table 3). *Dl*^{CS} is an allele of *Dl* that enhances the ectopic bristle phenotype in flies mutant for *Bearded* (*Brd*), which influences N signaling and, like *nvy*, does not show a genetic interaction with null alleles of *Dl* (Leviten and Posakony, 1996).

These data suggest that *nvy*$^+$ activity promotes the SOP fate by affecting a component of the N signaling pathway. Given that *nvy* is normally expressed in the SOP, that *nvy*$^+$ activity biases a cell to become an SOP, and that ectopic Nvy expression non-autonomously inhibits the SOP fate, we suggest that *Dl*, or a gene that promotes Dl activity, may be a target of *nvy*. To further address this possibility, we examined Dl protein levels in imaginal discs in which *nvy* activity was manipulated. In third instar imaginal discs, we failed to detect a change in Dl levels in either *nvy*$^-$ clones or in clones ectopically expressing Nvy (Fig. 2J and data not shown; we also did not observe any change in *N* expression, data not shown). However, we reasoned that a positive affect on Dl levels by Nvy may be indirect and transient and therefore difficult to detect (see Discussion). To circumvent these potential problems, we constructed a constitutive transcriptional activator form of Nvy (VP16-Nvy) by fusing Nvy to the potent transcriptional activator, VP16. Because Nvy is likely

Table 2
Position of SOPs relative to the boundaries of neutral clones and clones expressing Nvy in wing discs

	# SOPs touching	# SOPs 1–2 cells away	*n*	% SOPs touching
neutral clone	73	57	130	56.2
ectopic *nvy* clone	38	74	112	33.9**

SOPs located close to clones were scored if the SOP was touching the clone or if it was one to two cells away from the clone (*n* = number of SOPs scored). Significantly fewer SOPs were found touching the clones ectopically expressing *nvy* than neutral clones (**0.001 > *p*).

Table 3
Removing one copy of *nvy* enhances the *N* mutant phenotypes

	Ave. # SC	s.d.	*n*
wild type	4.00	0.00	142
nvy^{PDFKG1}/+	4.00	0.00	118
N^{XK11}/+	4.16	0.141	117
N^{XK11}/+; *nvy*^{PDFKG1}/+	4.58	0.738***	101
Dl^{B2}/+	4.00	0.00	101
nvy^{PDFKG1}/+; *Dl*^{B2}/+	4.00	0.00	117
Dl^{CS}/+	4.10	0.298	74
nvy^{PDFKG1}/+; *Dl*^{CS}/+	4.40	0.629**	91

Ectopic SC bristles form in *N*^{XK11} heterozygous flies. Removing one copy of *nvy* significantly enhanced the *N*^{XK11} phenotypes (**0.005 > *p*, ***0.0001 > *p*). *nvy* did not interact with other neurogenic genes (data not shown). s.d. = standard deviation; *n* = number of flies.

to be a transcriptional repressor (see below), VP16-Nvy is predicted to activate genes that Nvy normally represses. We also would predict that VP16-Nvy should have opposite consequences as Nvy. Consistent with these predictions, VP16-Nvy causes multiple SOPs to arise from the same PNC, opposite to the ectopic Nvy phenotype (Fig. 6E and see below). Moreover, VP16-Nvy caused a loss of Dl from the apical membrane and a decrease in vesicular Dl (Fig. 3B). Interestingly, VP16-Nvy did not alter the levels of a *Dl-lacZ* enhancer trap line (*Dl*$^{A326.2F3}$), suggesting that its affect on Dl protein levels is post-transcriptional (data not shown). Thus, assuming that VP16-Nvy behaves in a manner opposite to Nvy, these results suggest that Nvy promotes the SOP fate by increasing the amount of Dl the SOP produces. We suggest that our inability to directly observe a Nvy-induced increase in Dl levels is because this regulation is indirect and can only happen during a narrow window of development (see Discussion).

We tested the interaction between Nvy and the N pathway in an additional way. We reasoned that if Nvy was causing the bald phenotype by making all proneural cells better Dl signalers, Nvy should be unable to inhibit neurogenesis if the N pathway is blocked in receiving cells. To block N activity in receiving cells, we used a mutation in *nicastrin* (*nic*), which is required for N cleavage and, therefore, N-mediated gene regulation and neural fate repression (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and St. Johnston, 2002). On their own, *nic*$^-$ clones show de-repression of neural markers such as Sens (Chung and Struhl, 2001; Hu et al., 2002) (Fig. 3D). Ectopic Sens was also observed in *nic*$^-$ clones that are simultaneously forced to express Nvy (Fig. 3E). Thus, unlike in otherwise wild type cells, ectopic Nvy is unable to block neurogenesis when the N pathway cannot function, arguing that its effects are mediated by N pathway activation. These results are therefore consistent with the view that Nvy promotes N pathway activity by increasing Dl levels or activity. These results are also consistent with our observation that reducing the dose of wild type *nvy* enhanced a N loss-of-function phenotype (Table 3).

The ectopic Nvy phenotype can be rescued by increasing Da, but not Sc

Previous experiments support a model in which *ac* and *sc* enhance Dl signaling (Heitzler et al., 1996). Above, we show

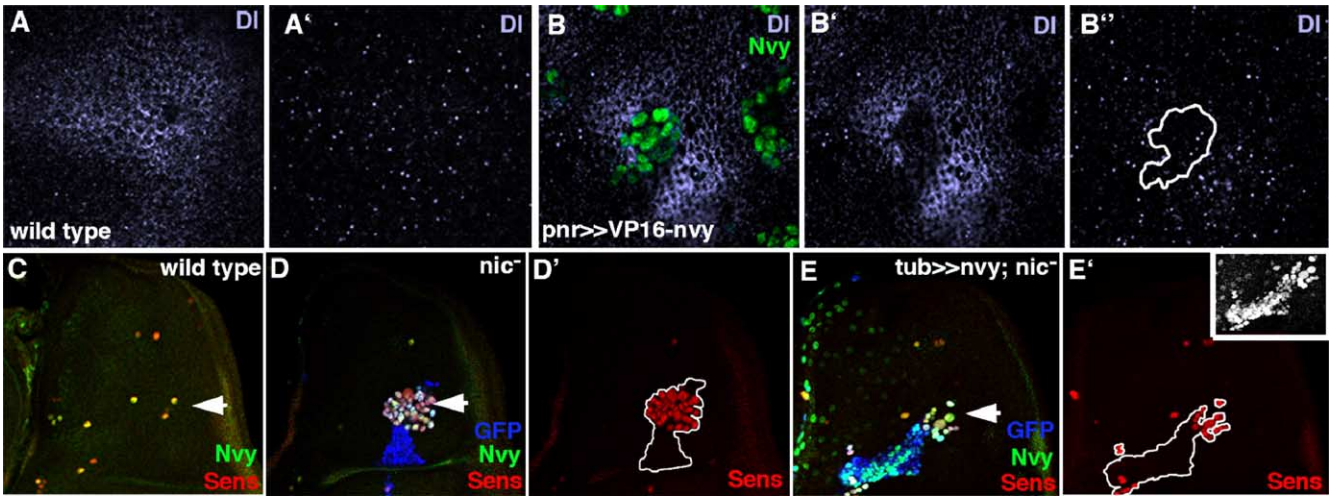

Fig. 3. Ectopic *nvy* acts downstream of N-Dl signaling. (A) Wild type expression of Dl in the DC PNC. An apical confocal section (left) shows the membrane localization of Dl and a more basal confocal section (A′, right) shows Dl localized in vesicles. (B) In clones expressing VP16-Nvy (*yw hs-flp*; *FRT40A tub-Gal80/ FRT40A*; *pnr-Gal4 UAS-VP16-nvy*) Dl is not present at the membrane (B, B′) and the amount of vesicular Dl is greatly reduced (B″). A similar effect on Dl was observed using *sca-Gal4* and *C96-Gal4* (a wing margin driver) to express VP16-Nvy (not shown). (C) Wild type wing disc stained for Nvy (green) and Sens (red) to mark the SOPs. Arrow indicates a group of SOPs (tr1, aPA, pSA) just ventral to the DC SOPs. (D) Ectopic SOPs form inside *nic*$^{-}$ clones (blue). The ectopic SOPs express Sens (red) and Nvy (green). The clone is outlined. (E) Ectopic Nvy (green) does not inhibit SOP formation in *nic*$^{-}$ clones (blue). SOPs are marked by Sens (red). The clone is outlined. The inset in E′ shows the Nvy channel; all cells in the clone express Nvy.

that *nvy* is expressed in the SOP after Ac and Sc levels accumulate. We also present results suggesting that *nvy* is able to promote Dl signaling. Together, these data suggest that *nvy* may act downstream of *ac* and *sc* to enhance the strength of the Dl signal. According to this view, the bald phenotype resulting from ectopic Nvy is due to an enhanced ability of all proneural cells to make active Dl. We tested this possibility by determining if ectopic *nvy* suppresses neurogenesis even when *sc* is over-expressed. Ectopic expression of Sc results in additional sensory organs (Fig. 4A). Co-expression of *nvy* with *sc* completely suppresses this phenotype, suggesting that *nvy* is able to block SOP formation even in the presence of high levels of Sc (Fig. 4C). Ectopic Nvy is also able to suppress the additional SOPs that form in response to ectopic Ase expression (Figs. 4E–F). Downstream of *ac* and *sc* is *sens*, which also causes additional sensory organs to develop when ectopically expressed (Fig. 4G). In this case, however, Nvy was unable to suppress the ectopic Sens phenotype (Fig. 4I). These results suggest that the suppression of SOP fate by Nvy occurs at a step downstream of Sc, but prior to Sens.

To bind DNA, Ac, Sc, and Ase require their dimerization partner Da (Cabrera and Alonso, 1991; Murre et al., 1989; Van Doren et al., 1991). As with *sc* and *ac* mutations, *da* mutant clones in the wing disc are devoid of sensory organs (Cadigan et al., 2002) (see Fig. 7A). The formation of other *da*-dependent sensory organs, such as the chordotonal organs (which form independently of *ac* and *sc*), are also blocked by ectopic Nvy (data not shown). Thus, the ectopic Nvy phenotype could be caused by Nvy interfering with Da. Ectopic Da expression promotes additional mechanosensory organs to form, although not as efficiently as Sc (Figs. 5C, G). However, unlike Sc, the expression of Da partially rescued the ectopic Nvy phenotype (Figs. 5D, H). In addition, reducing Nvy levels using *nvy-RNAi* or a *nvy* deficiency enhanced the extra sensory organ phenotype produced by ectopic Da (Table 4). As no affect on Da protein levels was observed (data not shown), these data suggest that ectopic Nvy interferes with the function of Da. We also found that Nvy and Da proteins physically interact. GST-Nvy was able to specifically pull-down Da from embryonic lysates and His-tagged Da (His-Da) from bacterial cell lysates (Figs. 5I, J).

Together, these results suggest that Nvy may function, at least in part, by binding Da and repressing gene expression. As it is unclear what targets, if any, Da homodimers regulate during SOP development, we further test this idea by determining if Nvy can transcriptionally repress Sc/Da targets.

Nvy directly inhibits E-lacZ expression

Nvy's ability to interact genetically and physically with Da prompted us to examine its ability to regulate enhancer elements that contain Sc/Da binding sites. The *sc* SOP enhancer, which is repressed by Nvy, contains two Sc/Da binding sites, called E boxes (Culi and Modolell, 1998). The first E box, E1, was multimerized and fused to *lacZ* to make *E-lacZ*, a reporter that drives expression throughout the PNC (Fig. 6A). When Sc or Da is ectopically expressed *E-lacZ* expression is expanded (Culi and Modolell, 1998; Giagtzoglou et al., 2003). Thus, the *E-lacZ* reporter functions as a read-out of Sc/Da activity.

In keeping with our prediction that Nvy acts a transcriptional repressor, Nvy repressed *E-lacZ* expression (Fig. 6B), suggesting that Nvy blocks Ac/Da and Sc/Da heterodimers from activating transcription. This result is consistent with data demonstrating that the mammalian homolog of Nvy, ETO, interacts with transcriptional repressors via domains that are conserved in Nvy (Davis et al., 2003). One way in which Nvy could inhibit *E-lacZ* expression is by associating with the

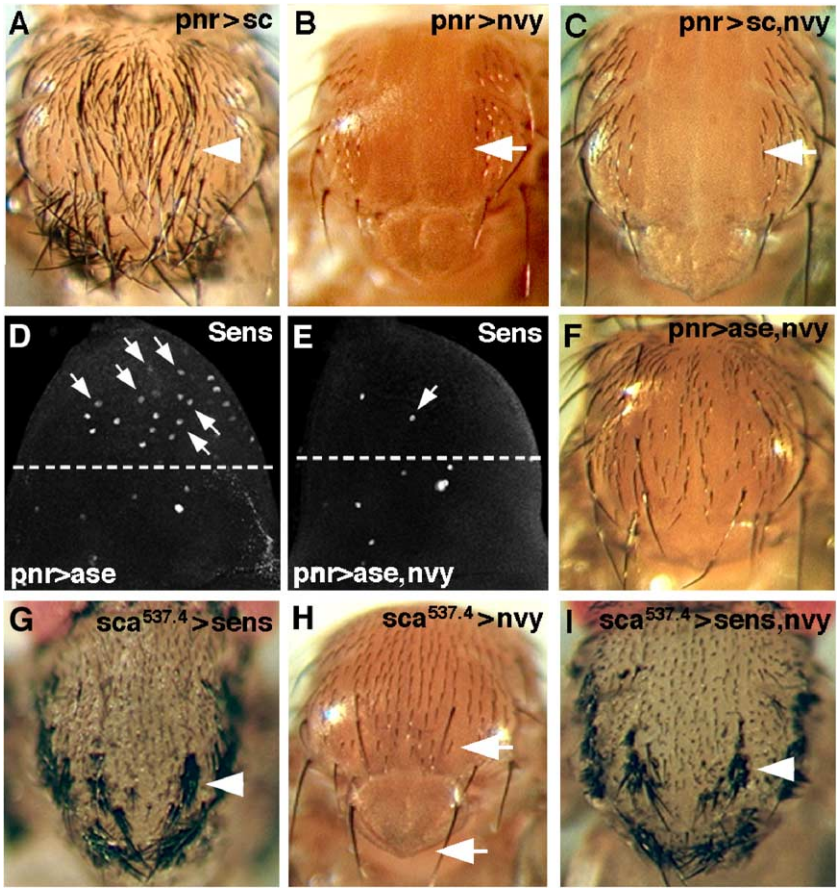

Fig. 4. Ectopic *nvy* acts downstream *ac*, but upstream of *sens*. (A–C, G–I) Arrowheads point to ectopic bristles and arrows indicate missing bristles. (A) Many macrochaetae form in *pnr-Gal4 UAS-sc* flies. (B) There are no sensory organs in the *pnr* domain of *pnr-Gal4 UAS-nvy* flies. (C) *pnr-Gal4 UAS-sc UAS-nvy* phenocopies *pnr-Gal4 UAS-nvy*. (D) Ectopic Ase (*pnr-Gal4 UAS-ase*) strongly stimulates SOP differentiation in the larval wing disc (Sens in white marks the SOPs, arrows) and causes flies to die during pupation. (E, F) *pnr-Gal4 UAS-ase UAS-nvy* flies display an intermediate phenotype: there are a few SOPs (arrow) in the larval wing disc (E) and some bristles in the adult (F). There are no SOPs in the *pnr-Gal4 UAS-nvy* larval wing discs (see Fig. 5F). (G) Ectopic Sens (sca^{C253}*-Gal4 UAS-sens*) results in tufts of bristles in the adult. (H) sca^{C253}*-Gal4 UAS-nvy* flies have missing bristles (arrows). (I) sca^{C253}*-Gal4 UAS-sens UAS-nvy* nota appear the same as sca^{C253}*-Gal4 UAS-sens* nota.

DNA, either directly or via an interaction with Da. Alternatively, by binding Da, Nvy could sequester Ac/Da and Sc/Da heterodimers away from DNA, preventing them from activating gene expression. There is also a report suggesting that Nvy has a non-nuclear function, raising the possibility that Nvy could act outside the nucleus (Terman and Kolodkin, 2004, but see Ice et al., 2005). We used VP16-Nvy to distinguish between these two scenarios. If Nvy functions off the DNA or outside the nucleus, VP16-Nvy should have similar activity to Nvy and repress *E-lacZ*. In contrast, if Nvy represses *E-lacZ* by associating with the DNA (either directly or indirectly), VP16-Nvy should activate *E-lacZ* and may result in flies with extra sensory organs.

Strikingly, ectopic VP16-Nvy activated *E-lacZ* and resulted in ectopic SOPs (Figs. 6C, E). *E-lacZ* expression was activated outside the endogenous *ac* and *sc* expression domains, suggesting that the effect of VP16-Nvy on *E-lacZ* does not only result from a change in N activity within the PNC. Further, the activation of *E-lacZ* by VP16-Nvy requires the E boxes present in *E-lacZ* to stimulate its expression because a reporter gene in which these binding sites are mutated (*mE-lacZ*) is not activated by VP16-Nvy (Culi and Modolell, 1998). These results suggest that Nvy affects Sc/Da activity by associating with DNA, perhaps via an interaction with Da.

We also tested if Nvy requires its zinc finger domain, which in ETO is known to recruit transcriptional co-repressors (Davis et al., 2003). Although the zinc finger domain is necessary for repression in cell culture (Lutterbach et al., 1998; Wang et al., 2004), removing the Nvy zinc fingers (NvyΔZF) had only a mild affect on Nvy activity in flies. *pnr-Gal4 UAS-nvyΔZF* flies are mostly bald, although the SC bristles and a few microchaetae develop normally (Fig. 6F). In addition, *E-lacZ* is not repressed in *pnr-Gal4 UAS-nvyΔZF* flies (data not shown). These results suggest that the zinc finger domain plays a role in Nvy's ability to suppress mechanosensory organ formation, but that other domains in Nvy can partially compensate in its absence.

VP16-Nvy phenocopies N loss-of-function independently of da

VP16-Nvy's ability to activate *E-lacZ*, but not *mE-lacZ*, together with our observation that Nvy interacts with Da, suggests that Nvy might affect gene expression by interacting with Ac/Da and Sc/Da in vivo. One test of this idea is to see if

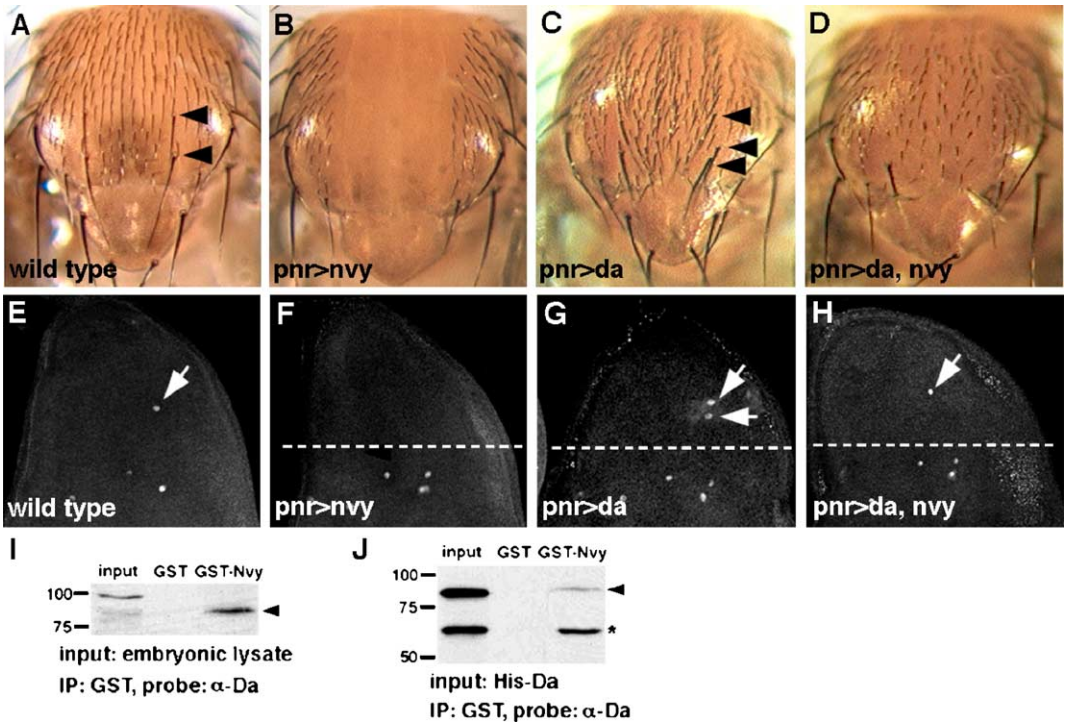

Fig. 5. Increased Da levels rescues Nvy-induced baldness and Nvy interacts with Da. Arrowheads indicate DC macrochaetae on the notum of adult flies (A–D). Larval wing discs (E–H) were stained with anti-Sens (white) to mark SOPs (arrows). (A, E) The back of a wild type fly (A) and a wild type larval wing disc (E). (B, F) There are neither bristles (B) nor SOPs (F) in the *pnr* domain of *pnr-Gal4 UAS-nvy* flies. (C, G) Ectopic Da (*pnr-Gal4 UAS-da*) results in ectopic macrochaetae (C) and SOPs (G). (D, H) *pnr-Gal4 UAS-da UAS-nvy* flies have macrochaetae (D) and SOPs (H). (I, J) GST and GST-Nvy bacterial cell lysates were incubated with embryonic lysate (I) or purified His-Da (J). The immunoblots were probed with anti-Da. The arrowheads indicate bands of the predicted Da size. The monoclonal Da antibody recognizes a background band in the embryonic lysate input lane (Brown et al., 1996). The asterisk indicates a truncated Da protein that also interacts with GST-Nvy. The input lane represents 1% of the starting material. The specificity of the anti-Da antibody was confirmed by showing that the same band indicated by the arrowhead increased in intensity when Da was overexpressed in vivo (data not shown).

VP16-Nvy requires *da* to activate *E-lacZ* expression, but we were unable to do this experiment for technical reasons. Instead, we asked if VP16-Nvy requires *da* to generate extra sensory organs. First, however, we better characterized the consequences of VP16-Nvy expression in the SOP lineage.

When the SOP divides, it produces two daughters that differ from both the SOP and each other. Due to the asymmetric distribution of regulatory factors, N is active in only one of the two daughters (see Fig. 1A). N activity promotes what is referred to as cell fate A (which includes the socket and sheath), and the absence of N activity results in cell fate B (which includes the bristle and neuron). Therefore, in the complete absence of *N* activity, not only do additional SOPs form (due to a failure in lateral inhibition), but the SOP progeny adopt B cell fates, resulting in mechanosensory organs with multiple neurons. Due to the absence of the external cell types, this cell fate transformation causes baldness. However, because of the additional neurons, the N^- bald phenotype is very different from the bald phenotype that results from ectopic Nvy expression, in which no SOPs are specified.

Table 4
Reducing *nvy* levels enhances the ectopic *da* phenotype

	Ave. # DC	s.d.	Ave. # SC	s.d.	*n*
wild type	4.00	0.00	4.01	0.117	146
pnr-Gal4UAS-nvy-RNAi	4.00	0.00	4.31	0.498	70
pnr-Gal4UAS-Da	4.04	0.283	4.32	0.653	50
pnr-Gal4UAS-Da UAS-nvy-RNAi	4.18	0.546	5.36	0.891***	45
In(2LR)Px^4; *pnr-Gal4UAS-Da*	4.00	0.371	4.50	0.777	30
In(2LR)Px^4; *pnr-Gal4UAS-Da UAS-nvy-RNAi*	4.33	0.555**	6.41	1.462***	27

pnr-Gal4 UAS-da flies have extra SC and DC macrochaetae. Reducing *nvy* levels, using either *UAS-nvy-RNAi* and/or a *nvy* deficiency (*In(2LR)Px*^{4}), significantly increases the number of extra SC and DC bristles when compared to *pnr-Gal4 UAS-Da* flies (**0.005 > *p*, ***0.0001 > *p*). s.d. = standard deviation; *n* = number of flies.

Expressing VP16-Nvy using *neur-Gal4*, which is restricted to the SOP and its progeny, resulted in lethality. Therefore, we used an *FRT tubulin-Gal80* chromosome to generate clones that express VP16-Nvy under the control of *neur-Gal4*. The *neur-Gal4 UAS-VP16-nvy* phenotype is dramatically different from *neur-Gal4 UAS-nvy*, which has no affect on mechanosensory organ development. Instead, expressing VP16-Nvy in the SOP lineage caused the SOP progeny to adopt a B cell fate, similar to *N* and *Dl* loss-of-function phenotypes (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993; Zeng et al., 1998). These sensory organs were comprised of multiple neurons and occasionally had more than one sheath cell (Fig. 6G). In the adult fly, clones of *neur-Gal4 UAS-VP16-nvy* expressing cells resulted in baldness, another indication that the

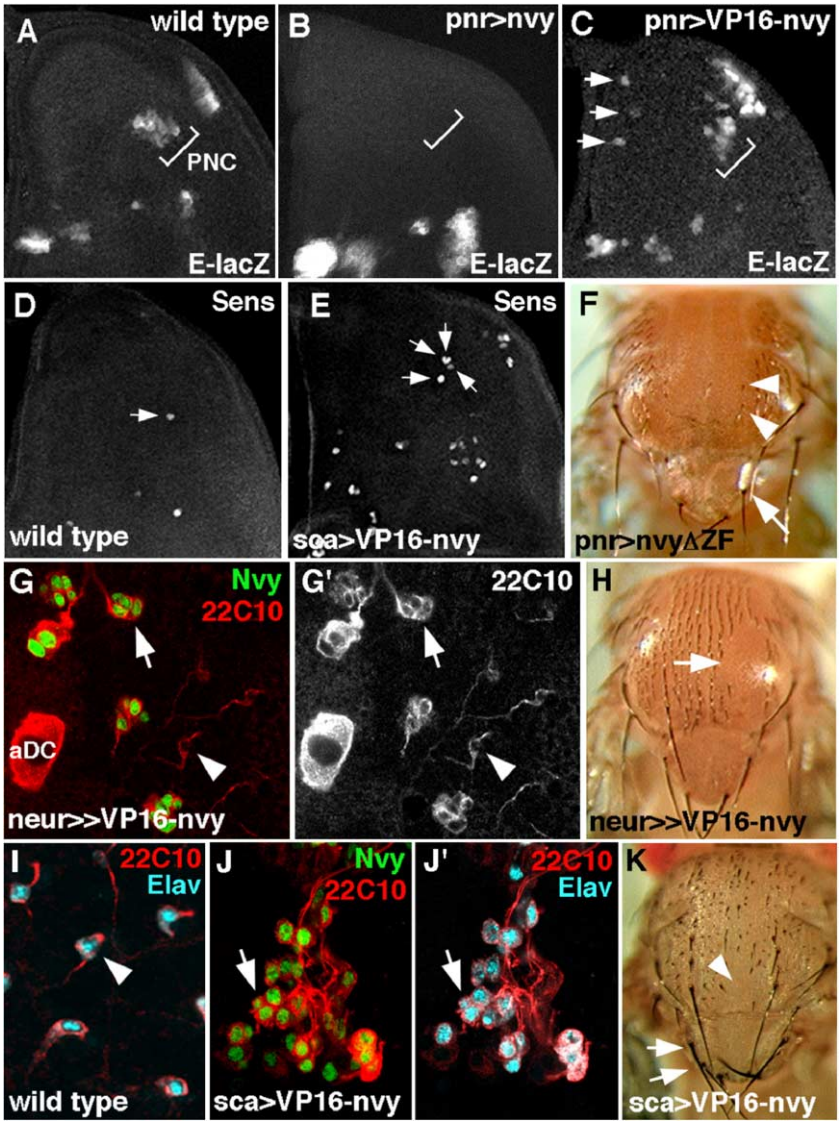

Fig. 6. Nvy inhibits, and VP16-Nvy stimulates, *E-lacZ* expression and neural development. Pupal nota (G, I, J) were stained for Nvy (green), 22C10 (red, white) and Elav (blue). 22C10 and Elav are neuronal markers. (A) Wild type expression pattern of *E-lacZ* (white) in the DC PNC (bracket). (B) *E-lacZ* expression (white) is inhibited in *pnr-Gal4 UAS-nvy* wing discs. (C) *pnr-Gal4 UAS-VP16-nvy* activates *E-lacZ* (white) in anterior wing disc cells (arrows). (D) Wild type larval wing disc stained with anti-Sens (white). (E) sca^{C253}*-Gal4 UAS-VP16-nvy* results in ectopic SOPs (arrows), marked by anti-Sens (white). (F) Only the SC macrochaetae (arrow) and occasional microchaetae (arrowhead) remain in *pnr-Gal4 UAS-nvyΔZF* flies. Expression of NvyΔZF also partially represses *sens* (not shown). (G) Clones expressing VP16-Nvy under the control of *neur-Gal4* (*yw hs-flp*; *tub-Gal80 FRT 40A*/*FRT 40A*; *neur-Gal4 UAS-VP16-nvy*) result in clusters of neurons (arrow). For comparison, wild type mechanosensory organs (arrowhead) have only one neuron. The large sensory organ is the aDC. The clones, marked by anti-Nvy, strongly express VP16-Nvy; endogenous Nvy is not visible using these antibody and confocal conditions. (H) In the adult, *neur-Gal4 UAS-VP16-nvy* clones (arrow) are bald. (I) A close-up of a wild type pupal notum. The arrowhead indicates an individual mechanosensory organ with a single neuron. (J) Large clusters of neurons are present in sca^{C253}*-Gal4 UAS-VP16-nvy* pupal notums. Individual mechanosensory organs cannot be distinguished. (K) sca^{C253}*-Gal4 UAS-VP16-nvy* adults have both extra mechanosensory organs (arrows) and bald areas (arrowhead). In this case, bald areas indicate a transformation of the external socket and bristle into neurons.

external components of the mechanosensory organ were transformed into internal components (Fig. 6H). Similar transformations occurred when VP16-Nvy was expressed with *sca-Gal4*. However, due to its activity within both the PNC and the SOP lineage, this driver resulted in both *N* loss-of-function phenotypes: ectopic SOPs (and sensory organs) and clusters of neurons (and baldness) (Figs. 6J, K). Thus, expression of VP16-Nvy, which presumably results in the activation of genes that are normally repressed by Nvy, results in N^{-} (and Dl^{-}) phenotypes even when its expression is restricted to the SOP lineage. These phenotypes are consistent with our observation that VP16-Nvy has the potential to repress Dl levels (see Fig. 3).

We then asked if VP16-Nvy's activity is *da*-dependent. Surprisingly, expressing VP16-Nvy in *da* mutant clones resulted in clusters of neurons and a decrease in Dl levels, similar to expressing VP16-Nvy in otherwise wild type animals (Figs. 7B–E and data not shown). The VP16-Nvy-positive, *da*-mutant clones also resulted in bald patches in the adult (Fig. 7D). *da* mutant clones in the adult were also bald, but this is due to a loss of SOPs rather than a cell fate transformation

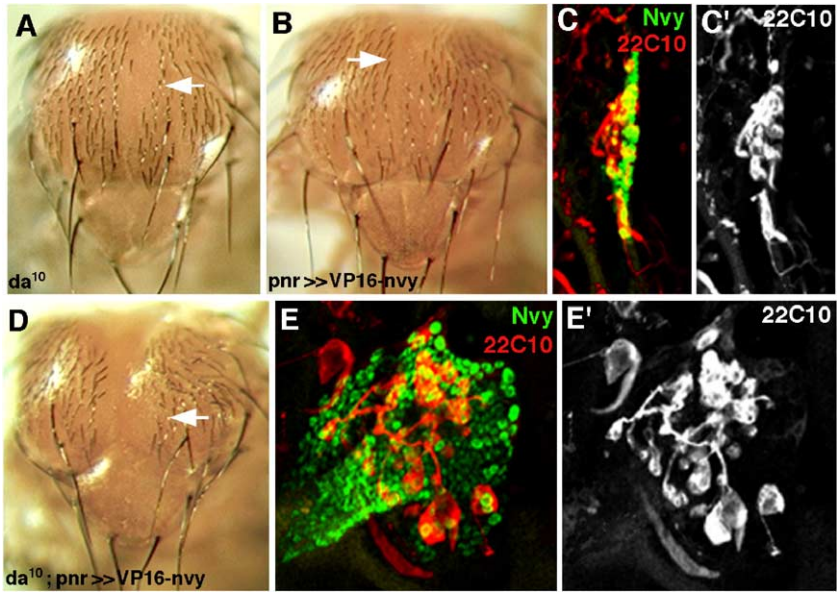

Fig. 7. VP16-Nvy promotes neural development in the absence of *da*. Pupal nota (C, E) were stained with anti-Nvy (green) and 22C10 (red or white). Arrows indicate the clones. (A) *da* loss-of-function clones produce bald patches (arrow) in the adult. (B, C) Clones expressing VP16-Nvy (*yw hs-flp*; *tub-Gal80 FRT 40A*/*FRT 40A*; *pnr-Gal4 UAS-VP16-nvy*) result in clusters of neurons in the pupal notum (C) and patches of deformed, bald cuticle in the adult (B). The gross increase in neural differentiation in the VP16-Nvy-expressing clones probably causes the cuticular deformities seen in the adult. (D, E) VP16-Nvy stimulates neural differentiation without *da* (*yw hs-flp*; *tub-Gal80 FRT 40A*/*da*^{10} *FRT 40A*; *pnr-Gal4 UAS-VP16-nvy*). In the pupal notum (E), there are clusters of neurons and the adult notum (D) has regions that are bald and deformed. These clones are also still able to repress Dl levels (not shown).

(Fig.7A). Thus, VP16-Nvy does not require *da* to decrease Dl levels, inhibit N signaling, and promote a neural fate.

Discussion

Nvy and Notch-Delta signaling

In the *Drosophila* PNS, sensory organ fate is promoted by *ac* and *sc* expression and antagonized by N activity. Once expressed, *ac* and *sc* are able to positively auto-regulate their expression via the SOP enhancer. N activity inhibits SOP enhancer activity, preventing the accumulation of Ac and Sc. In principle, for a PNC cell to accumulate more Ac and Sc than its neighbors, it would have to either receive a weaker Dl signal (resulting in less N activity autonomously) and/or send a stronger Dl signal. Genetic data support the idea of a feedback loop in which Ac and Sc increase the strength of the Dl signal sent by the presumptive SOP, which results in a decrease in Ac and Sc levels (and weaker Dl signal) in neighboring cells (Heitzler et al., 1996). Thus, it is hypothesized that a nascent SOP sends a stronger Dl signal than its neighbors and consequently receives a weaker Dl signal. This combination allows a PNC cell to achieve high Ac and Sc levels and adopt the SOP fate.

Although there is genetic evidence that Ac and Sc increase Dl signaling strength, it is not clear how Ac and Sc achieve this effect. In particular, an increase in Dl protein levels in the SOP has not been observed (Kooh et al., 1993), suggesting that Dl transcription is either not significantly increased in the SOP, or that changes in Dl levels are too transient and/or subtle to reliably detect. In contrast, Ac and Sc have been shown to directly increase Dl expression in the developing embryonic nervous system, suggesting that this form of regulation is also possible in the SOP (Kunisch et al., 1994). An alternative possibility, however, is that Ac and Sc could cause a change in Dl localization that enhances its ability to signal to neighboring PNC cells. Such a mechanism is consistent with several recent observations that suggest Dl localization and cycling within cells are important factors affecting its signaling potential (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001, 2005; Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003; Pavlopoulos et al., 2001; Wang and Struhl, 2004, 2005; Yeh et al., 2001). However, because they are transcriptional regulators, Ac and Sc would only be expected to indirectly cause a change in Dl localization.

Our results suggest that Nvy plays a role, albeit subtle, in the SOP's ability to send a strong Dl signal to neighboring cells. Although our data demonstrate that *nvy* is not required for the SOP fate, we suggest that its ability to increase the Dl signal sent by the SOP helps to reinforce the SOP fate. We first became interested in *nvy* because when it is ectopically expressed it completely inhibits the formation of mechanosensory organs. Using reagents that mark the PNC and SOP, we found that ectopic Nvy blocks the formation of the SOP, but not the PNC. In contrast, elevating Nvy levels specifically within the SOP (using *neur-Gal4*) does not affect sensory organ development, indicating that ectopic Nvy blocks the formation of the SOP but does not inhibit its development once it is specified. Furthermore, ectopic Nvy does not block mechanosensory organ formation when Sens is also over-expressed, suggesting that ectopic Nvy blocks SOP formation before there are high levels of Sens in the nascent SOP. Consistent with this idea, we do not observe Sens expression in the *pnr* domain of *pnr-Gal4 UAS-nvy* wing discs or in clones that ectopically

express Nvy. These data suggest that ectopic Nvy interferes with SOP formation at a stage before Sens is expressed, which corresponds to when the SOP is initially specified.

nvy is normally expressed in the SOP shortly after Ac and Sc levels increase. Given the expression of endogenous *nvy* within the SOP, we considered the following two possibilities to explain the ectopic Nvy phenotype and to gain some clues about wild type function of *nvy*. First, it is possible that ectopic Nvy blocks SOP formation cell autonomously by inhibiting the expression of *ac*, *sc*, or their downstream targets (such as *sens*) that are necessary for SOP formation. Second, it is possible that ectopic Nvy acts cell non-autonomously by enhancing Dl signaling, resulting in the 'mutual inhibition' of cells expressing precociously high levels of *nvy*. A closer examination of clones that ectopically express Nvy revealed that SOPs were significantly less likely to form near the borders of Nvy expressing clones than control clones. These results suggest that Nvy is acting, at least in part, cell non-autonomously, perhaps by increasing the strength of the Dl signal (we discuss the possibility that Nvy may also act cell autonomously in the following section). As a test of this idea, we ectopically expressed Nvy in clones lacking *nic*, which encodes a transmembrane protein required for cleaving and activating N in response to ligand binding (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and St. Johnston, 2002). Ectopic Nvy was unable to block SOP formation in *nic* mutant clones, demonstrating that Nvy's ability to block SOP formation requires the N signaling pathway to be intact. This finding is therefore consistent with the idea that Nvy normally enhances the level of active Dl in the SOP. Importantly, our loss-of-function *nvy* experiments are also consistent with this proposed role for Nvy. Using two different methods to remove *nvy* (expressing *nvy* RNAi or generating clones of a *nvy* deficiency), we found that PNC cells that neighbor nvy^- clones are more likely to adopt the SOP fate than PNC cells that neighbor wild type clones. This result is similar to what was observed when the relative amount of Dl differs between neighboring PNC cells: PNC cells that neighbor cells with less Dl are more likely to differentiate as SOPs (Heitzler and Simpson, 1991). In contrast to the Dl experiments, however, the complete absence of *nvy* did not cause all PNCs to become SOPs. Keeping in mind that *nvy* expression is restricted to the SOP (*nvy* is not detectably expressed in the PNC), these data suggest that *nvy* is not a general regulator of Dl signaling throughout the PNC, but that *nvy* enhances Dl activity in the SOP when it is forming.

Although these experiments are consistent with the idea that *nvy* enhances Dl signaling in the SOP, we were unable to directly detect changes in Dl protein levels in either *nvy* loss- or gain-of-function situations. There are several possible explanations for this negative result. First, it is possible that *nvy* does affect Dl expression levels, but that the change is too slight or brief to distinguish with the available anti-Dl antibody. Second, *nvy* might not affect Dl expression, but affect its localization and/or signaling ability in a manner that cannot be detected in our experiments. Third, it is also possible that *nvy* does not affect Dl at all, but interacts with other factors to produce the phenotypes we observe. We suggest that our experiments using VP16-Nvy help to distinguish between these possibilities. Expressing VP16-Nvy produces results opposite to those resulting from expressing Nvy: VP16-Nvy enhances *E-lacZ* expression, which ectopic Nvy represses, and its expression results in ectopic $Sens^+$ SOPs. Based on these data and the evidence that ETO, the mammalian homolog of Nvy, acts as a transcriptional repressor, we suggest that VP16-Nvy acts as a transcriptional activator of targets that wild type Nvy normally represses. When expressed in a PNC, VP16-Nvy strongly reduces the amount of Dl observed at the cell surface and in intracellular vesicles. This result suggests that wild type Nvy has the potential to affect Dl, although the result does not distinguish an effect on expression from an effect on protein stability or trafficking. That ectopic Nvy does not inhibit the expression of *Dl-lacZ* suggests that Nvy may be more likely to transcriptionally regulate a factor is involved in Dl stability or trafficking. Regardless of the mechanism, the finding that VP16-Nvy reduces Dl levels suggests that wild type Nvy has the potential to increase Dl levels, a proposal that is consistent with our other loss- and gain-of-function experiments.

The VP16-Nvy results, while consistent with the idea that Nvy affects Dl, do not explain why we failed to observe a change in Dl levels in *nvy* loss- and gain-of-function experiments. Thus, we return to the explanation proposed above: that Nvy causes a small and/or transient increase in Dl activity (by affecting its expression, stability or localization). As mentioned in the beginning of Discussion, a change in the amount or localization of Dl in wild type SOPs has not been observed (Kooh et al., 1993), despite genetic evidence that Dl signaling is a critical step in SOP fate determination. The lack of an observable change in Dl during wild type development, in combination with our findings, lead us to propose that the presumptive SOP may send a transient pulse of increased Dl signal that is sufficient to bias cell fates within the PNC. Nvy may, therefore, contribute to this transient pulse of Dl.

Nvy as a transcriptional repressor

The experiments described here also shed some light on the molecular activities Nvy has in the SOP. First, based on its ability to repress well-defined *lacZ* reporter genes, Nvy appears to be a transcriptional repressor, as is its mammalian homolog ETO. Second, we show that ectopic Nvy appears to interfere with the function (as opposed to the expression) of Ac and Sc because re-supplying Ac and Sc in *pnr-Gal4 UAS-nvy* flies was unable to rescue the bald phenotype. In contrast, expression of Da, a bHLH DNA binding partner for Ac and Sc, was able to partially rescue the bald phenotype of *pnr-Gal4 UAS-nvy* flies. Moreover, we found that *nvy* and *da* genetically interact (e.g., reducing *nvy* levels enhanced a *da* gain-of-function phenotype) and that Nvy and Da physically interact. These findings are consistent with a recent report showing that ETO directly interacts with HEB, a bHLH factor in the same class as Da (Zhang et al., 2004). The domain through which ETO interacts

with HEB (and other mammalian class I bHLH transcription factors) is conserved in Nvy, and HEB's ETO interaction domain is found in Da. These data lead us to propose that Nvy, a presumptive transcriptional repressor, has the ability to function with Ac/Da and Sc/Da heterodimers to repress the transcription of some target genes. In the absence of Nvy, such as in the non-SOP cells of a PNC, Ac/Da and Sc/Da may have the potential to activate these same target genes. However, our experiments also suggest that the interaction between Nvy and Da may not be required for all of Nvy's functions because VP16-Nvy was able to lower Dl levels even in *da* mutant clones. One potential explanation for this Da-independent function is that Nvy may be able to directly interact with DNA (J.W. and R.S.M. unpublished observations). In summary, we speculate that the Nvy–Da interaction is only required for the regulation of a subset of target genes.

Our proposal that Nvy works with Ac/Da and Sc/Da to repress target genes may on the surface seem at odds with our suggestion that Nvy can transiently increase the levels of Dl, because it is thought that Ac/Da and Sc/Da heterodimers activate *Dl* expression in the SOP. However, as described above, it is not known if Dl levels are in fact directly increased by Ac/Sc. Second, we stress that the timing of expression of these genes is critical to understanding how they function in vivo. Based on the wild type timing of its expression, *nvy* is likely to be a target of Ac/Sc in the presumptive SOP. Accordingly, there will be a window of time when Ac/Sc levels are high and Nvy levels are low in the presumptive SOP. This window of time may be sufficient for Ac/Sc to affect Dl expression and initiate the bias in favor of the SOP fate. Once Nvy levels increase, it may then work with Ac/Sc to repress the expression of some target genes, some of which may cause a further increase in Dl signaling. However, we hypothesize that *nvy*'s role in this process is after the bias has already been initiated.

In summary, we suggest that Nvy plays a subtle but observable role in the establishment of the SOP fate. Although it is not essential for the SOP fate, we suggest that Nvy helps the SOP/non-SOP bias by increasing the strength of the Dl signal sent by the SOP. Because *nvy* is evolutionarily conserved, both in its protein sequence and nervous system expression, we suggest that this role, although subtle, is important for the stereotyped uniformity of mechanosensory organ development. In addition, *nvy* may also play a role in later stages of neurogenesis, in particular axon pathfinding (Terman and Kolodkin, 2004). Because of Nvy's role as a transcriptional repressor, we further suggest that Nvy increases the Dl signal indirectly, by repressing a gene (factor X) that normally inhibits Dl activity. Based on Nvy's ability to interact with Da, this hypothetical target may be repressed by Nvy in combination with Ac/Da and Sc/Da heterodimers. Interestingly, it follows that in non-SOP cells of the PNC, which express *ac* and *sc* but not *nvy*, this hypothetical target may continue to be expressed, helping to downregulate Dl activity in these cells and thereby further increase the SOP/non-SOP bias. Clearly, the test of this proposal requires the identification of factor X as well as a more detailed understanding of how Dl levels and activity are modulated in the SOP.

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References

Bailey, A.M., Posakony, J.W., 1995. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. Genes Dev. 9, 2609–2622.

Brand, M., Jarman, A.P., Jan, L.Y., Jan, Y.N., 1993. asense is a Drosophila neural precursor gene and is capable of initiating sense organ formation. Development 119, 1–17.

Brown, N.L., Paddock, S.W., Sattler, C.A., Cronmiller, C., Thomas, B.J., Carroll, S.B., 1996. Daughterless is required for *Drosophila* photoreceptor cell determination, eye morphogenesis, and cell cycle progression. Dev. Biol. 179, 65–78.

Cabrera, C.V., Alonso, M.C., 1991. Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of *Drosophila*. EMBO J. 10, 2965–2973.

Cadigan, K.M., Jou, A.D., Nusse, R., 2002. Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless. Development 129, 3393–3402.

Chung, H.M., Struhl, G., 2001. Nicastrin is required for Presenilin-mediated transmembrane cleavage in *Drosophila*. Nat. Cell Biol. 3, 1129–1132.

Cubas, P., de Celis, J.F., Campuzano, S., Modolell, J., 1991. Proneural clusters of achaete–scute expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. Genes Dev. 5, 996–1008.

Culi, J., Modolell, J., 1998. Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. Genes Dev. 12, 2036–2047.

Davis, J.N., McGhee, L., Meyers, S., 2003. The ETO (MTG8) gene family. Gene 303, 1–10.

Deblandre, G.A., Lai, E.C., Kintner, C., 2001. *Xenopus* neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. Dev. Cell 1, 795–806.

Dominguez, M., Campuzano, S., 1993. asense, a member of the *Drosophila* achaete–scute complex, is a proneural and neural differentiation gene. EMBO J. 12, 2049–2060.

Feinstein, P.G., Kornfeld, K., Hogness, D.S., Mann, R.S., 1995. Identification of homeotic target genes in *Drosophila melanogaster* including nervy, a proto-oncogene homologue. Genetics 140, 573–586.

Fichelson, P., Gho, M., 2003. The glial cell undergoes apoptosis in the microchaete lineage of *Drosophila*. Development 130, 123–133.

Garcia-Garcia, M.J., Ramain, P., Simpson, P., Modolell, J., 1999. Different contributions of pannier and wingless to the patterning of the dorsal mesothorax of *Drosophila*. Development 126, 3523–3532.

Giagtzoglou, N., Alifragis, P., Koumbanakis, K.A., Delidakis, C., 2003. Two modes of recruitment of E(spl) repressors onto target genes. Development 130, 259–270.

Gomez-Skarmeta, J.L., Campuzano, S., Modolell, J., 2003. Half a century of neural prepatterning: the story of a few bristles and many genes. Nat. Rev., Neurosci. 4, 587–598.

Hartenstein, V., Posakony, J.W., 1990. A dual function of the Notch gene in *Drosophila* sensillum development. Dev. Biol. 142, 13–30.

Heitzler, P., Simpson, P., 1991. The choice of cell fate in the epidermis of *Drosophila*. Cell 64, 1083–1092.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C., Simpson, P., 1996. Genes of the enhancer of split and achaete–scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in *Drosophila*. Development 122, 161–171.

Hinz, U., Giebel, B., Campos-Ortega, J.A., 1994. The basic-helix–loop–helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. Cell 76, 77–87.

Hu, Y., Ye, Y., Fortini, M.E., 2002. Nicastrin is required for gamma-secretase cleavage of the *Drosophila* Notch receptor. Dev. Cell 2, 69–78.

Huang, F., Dambly-Chaudiere, C., Ghysen, A., 1991. The emergence of sense organs in the wing disc of *Drosophila*. Development 111, 1087–1095.

Ice, R., Wildonger, J., Mann, R., Hiebert, S., 2005. Comment on "Nervy links protein kinase a to plexin-mediated semaphorin repulsion". Science 309, 558b.

Itoh, M., Kim, C.H., Palardy, G., Oda, T., Jiang, Y.J., Maust, D., Yeo, S.Y., Lorick, K., Wright, G.J., Ariza-McNaughton, L., Weissman, A.M., Lewis, J., Chandrasekharappa, S.C., Chitnis, A.B., 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev. Cell 4, 67–82.

Jafar-Nejad, H., Acar, M., Nolo, R., Lacin, H., Pan, H., Parkhurst, S.M., Bellen, H.J., 2003. Senseless acts as a binary switch during sensory organ precursor selection. Genes Dev. 17, 2966–2978.

Jarman, A.P., Brand, M., Jan, L.Y., Jan, Y.N., 1993. The regulation and function of the helix–loop–helix gene, asense, in *Drosophila* neural precursors. Development 119, 19–29.

Jennings, B., Preiss, A., Delidakis, C., Bray, S., 1994. The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the *Drosophila* embryo. Development 120, 3537–3548.

Kalidas, S., Smith, D.P., 2002. Novel genomic cDNA hybrids produce effective RNA interference in adult *Drosophila*. Neuron 33, 177–184.

Kooh, P.J., Fehon, R.G., Muskavitch, M.A., 1993. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during *Drosophila* development. Development 117, 493–507.

Kunisch, M., Haenlin, M., Campos-Ortega, J.A., 1994. Lateral inhibition mediated by the *Drosophila* neurogenic gene delta is enhanced by proneural proteins. Proc. Natl. Acad. Sci. U. S. A. 91, 10139–10143.

Lai, E.C., Deblandre, G.A., Kintner, C., Rubin, G.M., 2001. *Drosophila* neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. Dev. Cell 1, 783–794.

Lai, E.C., Roegiers, F., Qin, X., Jan, Y.N., Rubin, G.M., 2005. The ubiquitin ligase *Drosophila* Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. Development 132, 2319–2332.

Le Borgne, R., Schweisguth, F., 2003. Notch signaling: endocytosis makes delta signal better. Curr. Biol. 13, R273–R275.

Le Borgne, R., Remaud, S., Hamel, S., Schweisguth, F., 2005. Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in *Drosophila*. PLoS Biol. 3, e96.

Lecourtois, M., Schweisguth, F., 1995. The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. Genes Dev. 9, 2598–2608.

Leviten, M.W., Posakony, J.W., 1996. Gain-of-function alleles of Bearded interfere with alternative cell fate decisions in *Drosophila* adult sensory organ development. Dev. Biol. 176, 264–283.

Lopez-Schier, H., St. Johnston, D., 2002. Drosophila nicastrin is essential for the intramembranous cleavage of notch. Dev. Cell 2, 79–89.

Lutterbach, B., Sun, D., Schuetz, J., Hiebert, S.W., 1998. The MYND motif is required for repression of basal transcription from the multidrug resistance 1 promoter by the t(8;21) fusion protein. Mol. Cell. Biol. 18, 3604–3611.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., et al., 1989. Interactions between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58, 537–544.

Nolo, R., Abbott, L.A., Bellen, H.J., 2000. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. Cell 102, 349–362.

Parks, A.L., Muskavitch, M.A., 1993. Delta function is required for bristle organ determination and morphogenesis in *Drosophila*. Dev. Biol. 157, 484–496.

Pavlopoulos, E., Pitsouli, C., Klueg, K.M., Muskavitch, M.A., Moschonas, N.K., Delidakis, C., 2001. Neuralized encodes a peripheral membrane protein involved in delta signaling and endocytosis. Dev. Cell 1, 807–816.

Rooke, J.E., Xu, T., 1998. Positive and negative signals between interacting cells for establishing neural fate. BioEssays 20, 209–214.

Simpson, P., 1997. Notch signalling in development: on equivalence groups and asymmetric developmental potential. Curr. Opin. Genet. Dev. 7, 537–542.

Terman, J.R., Kolodkin, A.L., 2004. Nervy links protein kinase a to plexin-mediated semaphorin repulsion. Science 303, 1204–1207.

Van Doren, M., Ellis, H.M., Posakony, J.W., 1991. The *Drosophila* extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete–scute protein complexes. Development 113, 245–255.

Wang, W., Struhl, G., 2004. *Drosophila* Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development 131, 5367–5380.

Wang, W., Struhl, G., 2005. Distinct roles for mind bomb, neuralized and epsin in mediating DSL endocytosis and signaling in *Drosophila*. Development 132, 2883–2894.

Wang, J., Wang, M., Liu, J.M., 2004. Domains involved in ETO and human N-CoR interaction and ETO transcription repression. Leuk. Res. 28, 409–414.

Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C.J., Boulianne, G.L., 2001. Neuralized functions as an E3 ubiquitin ligase during *Drosophila* development. Curr. Biol. 11, 1675–1679.

Zeng, C., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1998. Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the *Drosophila* sensory organ lineage. Genes Dev. 12, 1086–1091.

Zhang, J., Kalkum, M., Yamamura, S., Chait, B.T., Roeder, R.G., 2004. E protein silencing by the leukemogenic AML1–ETO fusion protein. Science 305, 1286–1289.